

RESEARCH ARTICLE

Molecular Genetics of Tetrahydrobiopterin-Responsive Phenylalanine Hydroxylase Deficiency

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Mutations in the phenylalanine hydroxylase (PAH) gene result in phenylketonuria (PKU). Tetrahydrobiopterin (BH₄)-responsive hyperphenylalaninemia has been recently described as a variant of PAH deficiency caused by specific mutations in the PAH gene. It has been suggested that BH₄-responsiveness may be predicted from the corresponding genotypes. Data from BH₄ loading tests indicated an incidence of BH₄-responsiveness of >40% in the general PKU population and >80% in mild PKU patients. The current project entailed genotype analysis of 315 BH₄-responsive patients tabulated in the BIOPKUdb database and comparison with the data from the PAHdb locus-specific knowledgebase, as well as with previously published PAH mutations for several European countries, Northern China, and South Korea. We identified 57 mutations, presenting with a substantial residual PAH activity (average ~47%), presumed to be associated with BH₄-responsiveness. More than 89% of patients are found to be compound heterozygotes. The three most common mutations found in >5% of BH₄-responsive patients are p.A403 V, p.R261Q, and p.Y414C. Using the Hardy-Weinberg formula the predicted average frequency of BH₄-responsiveness in European populations was calculated to be 55% (range 17–79%, lowest in Baltic countries and Poland and highest in Spain), 57% in Northern China, and 55% for South Korea. The genotype-predicted prevalence of BH₄-responsiveness was higher than prevalence data obtained from BH₄ loading tests. Inconsistent results were observed for mutations p.L48S, p.I65 T, p.R158Q, p.R261Q, and p.Y414C. Our data suggest that BH₄-responsiveness may be more common than assumed and to some extent may be predicted or excluded from the patient's genotype. *Hum Mutat* 29(1), 167–175, 2008. © 2007 Wiley-Liss, Inc.

KEY WORDS: phenylketonuria; PKU; PAH; hyperphenylalaninemia; BH₄

INTRODUCTION

Phenylketonuria (PKU; MIM# 261600) is an autosomal recessive genetic disorder caused by a deficiency of the hepatic phenylalanine-4-hydroxylase (PAH; EC 1.14.16.1) [Scriver and Kaufman, 2001]. PAH is a mixed function oxidase that catalyzes hydroxylation of phenylalanine (Phe) to tyrosine, the rate-limiting step in phenylalanine catabolism. The reaction is dependent on BH₄ as cofactor, molecular oxygen, and iron. PAH deficiency causes hyperphenylalaninemia (HPA) of variable degree. A distinction is made between PKU, which requires a phenylalanine-restricted diet, and mild hyperphenylalaninemia (MHP), a variant that does not require treatment. Depending on the phenylalanine tolerance, PKU may be divided into mild PKU, moderate PKU, and classical PKU [Guldberg et al., 1998; Kayaalp et al., 1997]; some centers only distinguish between mild and classical PKU. The PAH-mutation genotype is the main determinant of the metabolic phenotype. In 1999, Kure et al. [1999] described patients who responded to oral administration of BH₄ by lowering their blood phenylalanine levels, and subsequently several groups documented that BH₄ can be successfully used for the long-term treatment of HPA patients as an alternative

to a dietary treatment [Bélanger-Quintana et al., 2005; Cerone et al., 2004; Hennermann et al., 2005; Lambroschini et al., 2005; Muntau et al., 2002; Shintaku et al., 2004; Steinfeld et al., 2004; Trefz et al., 2001, 2005]. It has been estimated that, depending on the criteria selected, more than 30% of all HPA patients respond to BH₄ (20 mg/kg) [Bernegger and Blau, 2002] and that the main target for BH₄ administration are patients with mild to moderate PKU [Fiege and Blau, 2007].

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Molecular mechanisms responsible for BH₄-responsiveness in patients with PKU are multifactorial [Aguado et al., 2006; Blau and Erlandsen, 2004; Erlandsen et al., 2003, 2004; Kure et al., 2004; Pey et al., 2004; Steinfeld et al., 2003; Thöny et al., 2004] and depend on mutations in the *PAH* gene. Chaperon-like activity of BH₄ and stabilization of PAH protein and a considerable residual activity as well as an increase in intracellular BH₄ concentrations required for the full enzyme activity are some of the proposed mechanisms. Mutations so far described in patients with BH₄-responsive HPA are tabulated in the BIOPKU database (BIOPKUdb) and many of them have been shown to be associated with a significant residual PAH activity, when recombinantly expressed in different cell systems.

To estimate the frequency of BH₄-responsiveness in different populations based on genotype information, we calculated the allele and genotype frequencies for the BIOPKUdb and compared them with data published for different countries, including the PAHdb locus knowledgebase [Scriver et al., 2003].

PATIENTS AND METHODS

Source of Data

Data from 315 patients who responded to the BH₄ loading test (10–20 mg/kg) by lowering their blood phenylalanine levels by >30% after 8 to 24 hr, were tabulated in the International Database of Patients and Mutations Causing BH₄-Responsive HPA/PKU (BIOPKUdb; www.bh4.org/BH4DatabasesBiopku.asp; programmed in Access 2003, Microsoft, Redmond, WA). The minimal requirement for inclusion in the database was a 30% blood phenylalanine reduction 8 hr after administration of 10 mg/kg BH₄. It is assumed that patients loaded with 20 mg/kg BH₄ over 24 hr would have the same or even better responsiveness. Therefore, data were not stratified in separate groups. Dietary intake of phenylalanine and initial blood phenylalanine levels were different, depending on the actual phenotype. BIOPKUdb includes detailed information on genotype (mutation name, systemic name, location in gene, PAH domain, residual activity, and HPA type), BH₄ loading test data (amount of BH₄ used and percentage of responsiveness), and references.

Both protein and cDNA mutation names follow standard nomenclature recommendations (www.hgvs.org/mutnomen) with nucleotide +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, and the initiation codon denoted codon 1. Reference sequences: GenBank cDNA = NM_000277.1; protein = NP_000268.1.

PAHdb (www.pahdb.mcgill.ca; a locus-specific knowledgebase) [Scriver et al., 2006] tabulates a total of 3,137 alleles (regardless of BH₄-responsiveness) from countries all over the world. In 633 patients the genotype is defined (last access April 2007). In addition to molecular data, information about the source of alleles, polymorphic haplotype backgrounds, and effect of the allele on the enzyme activity is provided. Little information is provided about BH₄-responsiveness.

Allele frequencies of common PKU mutations in Europe were previously published [Zschocke and Hoffmann, 1999]; we included studies only if the mutation detection rate exceeded 80%. Full genotype data were available for 119 patients from Northern Ireland (60 different genotypes) [Zschocke et al., 1995] and 275 patients from Germany (165 different genotypes) [Zschocke and Hoffmann, 1999]. Information on BH₄-responsiveness was not available for those cohorts. In addition to European patients we also included published patients from Korea and China with fully characterized genotypes in our study. There

were 58 patients with 48 different genotypes from Korea (the original study comprised 79 patients, but achieved only a mutation detection rate of 75%) [Lee et al., 2004]. There were 155 patients with 97 different genotypes from China (the original study comprised 185 patients and achieved a mutation detection rate of 94%) [Song et al., 2005].

Data Limitation

Excluded from the study are patients from the BIOPKUdb with only one allele detected (13 patients) and those with two null alleles (no residual activity) (three patients).

Criteria for BH₄-Responsiveness

The criteria of BH₄-responsiveness in patients with PAH deficiency have been previously described [Blau and Erlandsen, 2004]; briefly, patients were classified as BH₄-responsive if they responded to the oral administration of BH₄ (10–20 mg/kg body weight) by lowering their blood Phe levels by at least 30% within 8 to 24 hr. A mutation/an allele was regarded as associated with BH₄-responsiveness if it was present either in homozygous form or compound heterozygous associated with a known null mutation. Mutations were also regarded as null mutations when in vitro residual activity was below 10% and the second mutation in a compound heterozygous BH₄-responsive patient had a substantial residual PAH activity (>10%). In vitro enzymatic activities (mutant PAH as a percentage of wild-type PAH) are generally higher than those reported in liver biopsies [Waters et al., 1998].

Alleles that could not be classified using these criteria were labeled unclear.

Population Genetics

To estimate the frequency of BH₄-responsiveness in different populations we regarded BH₄-responsive mutations as dominant in compound heterozygous patients. BH₄-responsiveness was expected in all patients with a BH₄-responsive mutation on at least one PAH gene copy. For populations without available genotypes, the expected frequency of genotypes associated with BH₄-responsiveness was estimated using the Hardy-Weinberg formula, as follows. We first calculated the combined allele frequencies for BH₄-responsive mutations and nonresponsive mutations (including unclear alleles), respectively. Subsequently, squared combined frequency of nonresponsive alleles was taken as the frequency of nonresponsive genotypes, while the other genotypes were regarded as BH₄-responsive.

RESULTS

BIOPKUdb

The BIOPKUdb comprises genotype information from 315 BH₄-responsive patients. 114 different PAH gene mutations were found, 57% of which were present in more than one patient (Table 1). About 50% of the mutations described in the BIOPKUdb were previously expressed recombinantly in eukaryotic cell systems or in *E. coli*, and many were found to have substantial residual activity (>10%). The average residual PAH activity calculated for the 40 most common BH₄-responsive mutations was 46.8% (data not shown).

Using the criteria outlined, 57 mutations were classified as BH₄-responsive (Fig. 1), 20 were classified as nonresponsive, and 37 mutations were termed unclear. The most common BH₄-responsive mutations in the database are p.A403V (56 alleles), p.R261Q (49 alleles), p.Y414C (44 alleles), p.A300S and p.V245A (26 alleles each), p.L48S (24 alleles), p.E390G (23 alleles), and

TABLE 1. Summary of the BIOPKUdb Mutations: Allele Frequency, Location in the Protein, and Residual PAH Activity*

Allele	Nucleotide aberration	Number of alleles (AF) (%)	Residual activity ^a	Domain (CBR) (AS)
BH₄-responsive mutations				
p.A403V	c.1208C>T	56 (8.9)	32	Catalytic
p.R261Q	c.782G>A	49 (7.8)	38.5	Catalytic (CBR1)
p.Y414C	c.1241A>G	44 (7)	36	Tetramerization
p.A300S	c.898G>T	26 (4.1)	31	Catalytic
p.V245A	c.734T>C	26 (4.1)	50	Catalytic (CBR1) (AS)
p.L48S	c.143T>C	24 (3.8)	39	Regulatory
p.E390G	c.1169A>G	23 (3.7)	72.5	Catalytic
p.R241C	c.721C>T	20 (3.2)	25	Catalytic
p.I65T	c.194T>C	18 (2.9)	25.3	Regulatory
p.R158Q	c.473G>A	14 (2.2)	10	Catalytic
p.V388M	c.1162G>A	14 (2.2)	27.5	Catalytic
p.D415N	c.1243G>A	8 (1.3)	93	Tetramerization
p.R408Q	c.1223G>A	7 (1.1)	49.7	Catalytic
p.R243Q	c.728G>A	6 (1.0)	23	Catalytic
p.R413P	c.1238G>C	6 (1.0)	66	Tetramerization
IVS4–5c>g	c.442–5C>G	5 (0.8)	?	Intronic
p.E178G	c.533A>G	5 (0.8)	39	Catalytic
p.F39del	c.115_117delTTC	5 (0.8)	20	Regulatory
p.F39L	c.117C>G	5 (0.8)	49	Regulatory
p.R68S	c.204A>T	5 (0.8)	87	Regulatory
p.A395P	c.1183G>C	4 (0.6)	15.5	Catalytic
p.L348V	c.1042C>G	4 (0.6)	41	Catalytic
p.M276V	c.826A>G	4 (0.6)	?	Catalytic
p.P211T	c.631C>A	4 (0.6)	72	Catalytic
p.P407S	c.1219C>T	4 (0.6)	94	Catalytic
p.R241H	c.722G>A	4 (0.6)	23	Catalytic
p.Y417H	c.1249T>C	4 (0.6)	?	Tetramerization
IVS10–3c>t	c.1066–3C>T	3 (0.5)	?	Intronic
p.A104D	c.311C>A	3 (0.5)	26	Regulatory
p.A309V	c.926C>T	3 (0.5)	44	Catalytic
p.K320N	c.960G>C	3 (0.5)	?	Catalytic
p.T380M	c.1139C>T	3 (0.5)	?	Catalytic (AS)
p.V230I	c.688G>A	3 (0.5)	63	Catalytic
p.D222G	c.665A>G	2 (0.3)	?	Catalytic
p.H170D	c.508C>G	2 (0.3)	43	Catalytic
p.I94del	c.283_285delATC	2 (0.3)	27	Regulatory
p.I94S	c.281T>G	2 (0.3)	?	Regulatory
p.T92I	c.275C>T	2 (0.3)	76	Regulatory
p.V190A	c.569T>C	2 (0.3)	110	Catalytic
p.A313T	c.937G>A	1 (0.2)	76	Catalytic
p.A373T	c.1117G>A	1 (0.2)	56	Catalytic
p.D129G	c.386A>G	1 (0.2)	?	Regulatory
p.D338Y	c.1012G>T	1 (0.2)	?	Catalytic
p.E76G	c.227A>G	1 (0.2)	47	Regulatory
p.I269L	c.805A>C	1 (0.2)	?	Catalytic
p.L308F	c.922C>T	1 (0.2)	49	Catalytic
p.L41F	c.121C>T	1 (0.2)	10	Regulatory
p.P122Q	c.365C>A	1 (0.2)	22	Regulatory
p.P147S	c.439C>T	1 (0.2)	?	Catalytic
p.P244L	c.731C>T	1 (0.2)	51	Catalytic
p.P275S	c.823C>T	1 (0.2)	?	Catalytic
p.P314S	c.940C>T	1 (0.2)	?	Catalytic
p.S110L	c.329C>T	1 (0.2)	?	Regulatory
p.S310Y	c.929C>A	1 (0.2)	?	Catalytic
p.S87R	c.261C>A	1 (0.2)	82	Regulatory
p.R176L	c.527G>T	1 (0.2)	31.5	Catalytic
p.V177M	c.529G>A	1 (0.2)	?	Catalytic
Unclear mutations				
IVS10–11g>a	c.1066–11G>A	27 (4.3)	?	Intronic
IVS4+5g>t	c.441+5G>T	5 (0.8)	?	Intronic
IVS2+5g>c	c.168+5G>C	4 (0.6)	?	Intronic
p.F55>Lfs	c.165delT	4 (0.6)	?	Regulatory
IVS7+1g>a	c.842+1G>A	3 (0.5)	?	Intronic
p.P362T	c.1084C>A	3 (0.5)	?	Catalytic
IVS1+5g>t	c.60+5G>T	2 (0.3)	?	Intronic
IVS4+4g>a	c.441+4A>G	2 (0.3)	?	Intronic
IVS4–1g>a	c.442–1G>A	2 (0.3)	?	Intronic
IVS10+1g>a	c.1065+1G>A	2 (0.3)	?	Intronic
p.F55del	c.163_165delTTT	2 (0.3)	?	Regulatory
p.F55L	c.165T>G	2 (0.3)	?	Regulatory
p.I95_K96delinsK	c.284_286delTCA	2 (0.3)	?	Regulatory
p.R53H	c.158G>A	2 (0.3)	?	Regulatory
IVS1+5g>a	c.60+5G>A	1 (0.2)	?	Intronic
IVS10del546	?	1 (0.2)	?	Intronic
IVS2–13T>g	c.IVS2–13T>G	1 (0.2)	?	Intronic
IVS3–22G>a	c.353–22G>A	1 (0.2)	?	Intronic
IVS7+5g>a	c.842+5G>A	1 (0.2)	?	Intronic
p.A132V	c.395C>T	1 (0.2)	?	Regulatory
p.G188D	c.563G>A	1 (0.2)	?	Catalytic
p.H201Y	c.601C>T	1 (0.2)	?	Catalytic
p.I65S/p.H170Q	c.194T>G/c.510T>A	1 (0.2)	?	Regulatory/catalytic

TABLE 1. Continued

Allele	Nucleotide aberration	Number of alleles (AF) (%)	Residual activity ^a	Domain (CBR) (AS)
p.L65V	c.193A>G	1 (0.2)	?	Regulatory
p.L287V	c.859C>G	1 (0.2)	?	Catalytic
p.L367>Pfs	c.1099_1100insC	1 (0.2)	?	Catalytic
p.P119S	c.355C>T	1 (0.2)	?	Regulatory
p.P275L	c.824C>T	1 (0.2)	?	Catalytic
p.P275R	c.824C>G	1 (0.2)	?	Catalytic
p.P281A	c.841C>G	1 (0.2)	?	Catalytic (CBR2)
p.P366H	c.1097C>A	1 (0.2)	?	Catalytic
p.R158W	c.472C>T	1 (0.2)	?	Catalytic
p.S16>XfsX1	c.47_48delCT	1 (0.2)	?	Regulatory
p.S67P	c.199T>C	1 (0.2)	?	Regulatory
p.T193I	c.578C>T	1 (0.2)	?	Catalytic
p.Y168H	c.502T>C	1 (0.2)	?	Catalytic
p.Y386C	c.1157A>G	1 (0.2)	?	Catalytic
BH₄-nonresponsive mutations				
p.R408W	c.1222C>T	35 (5.6)	1.85	Catalytic
IVS12+1g>a	c.1315+1G>A	14 (2.2)	0	Intronic
p.P281L	c.842C>T	12 (1.9)	1	Catalytic (CBR2)
p.R261X	c.781C>T	7 (1.1)	1	Catalytic (CBR1)
p.S349P	c.1045T>C	6 (1)	1	Catalytic
p.G272X	c.814G>T	4 (0.6)	1	Catalytic
p.R252W	c.754C>T	4 (0.6)	1	Catalytic (CBR1)
p.R243X	c.727C>T	3 (0.5)	1	Catalytic
p.A259T	c.775G>A	2 (0.3)	0.3	Catalytic (CBR1)
p.E280K	c.838G>A	2 (0.3)	1.95	Catalytic (CBR2)
p.K274_Y277>Nfs	c.822_832del11	2 (0.3)	?	Catalytic
p.R176X	c.526C>T	2 (0.3)	1	Catalytic
p.Y356X	c.1068C>G	2 (0.3)	1	Catalytic
p.D222>Efs	c.663_664delAG	1 (0.2)	1	Catalytic
p.L311P	c.932T>C	1 (0.2)	1	Catalytic
p.R111X	c.331C>T	1 (0.2)	1	Regulatory
p.R270K	c.809G>A	1 (0.2)	2.1	Catalytic
p.S411X	c.1232C>A	1 (0.2)	1	Tetramerization
p.T278I	c.833C>T	1 (0.2)	1	Catalytic
p.W187X	c.561G>A	1 (0.2)	0	Catalytic

^aBoth protein and cDNA numbers follow standard recommendations with nucleotide +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, and the initiation codon denoted codon 1. Reference sequences: GenBank cDNA = NM_000277.1; protein = NP_000268.1.

^bAverage PAH activity (%) calculated for different cell systems.

AF, allele frequency; CBR, cofactor binding region; AS, active site.

p.R241C (20 alleles) (Table 1). Of 315 patients, 32 were homozygous, 102 had two different BH₄-responsive alleles, and 173 had one BH₄-responsive allele and one either non-responsive or unclear allele. Eight patients (2.5%) were compound heterozygous for two unclear alleles. The most common BH₄-responsive genotypes are listed in Supplementary Table S1 (available online at <http://www.interscience.wiley.com/jpages/1059-7794/suppmat>).

The mutations collected in the BIOPKUdb are spread all over the PAH gene (Fig. 1). Only two out of 57 BH₄-responsive mutations were located in the cofactor binding domain CBR1 (p.V245A and p.R261Q). The overall distribution of the different mutations (responsive and nonresponsive) tabulated in the BIOPKUdb is as follows: 64% are found in the catalytic domain, 14% are in the regulatory domain, 10% in the tetramerization domain, and 12% are intronic. In comparison to nonresponsive mutations, responsive mutations are located with an increased percentage in the tetramerization (14% vs. 1%) and the regulatory domains (16% vs. 1%). Most of the responsive mutations were found in the catalytic domain (68%) (Table 2). There was a small number of splicing mutations that are BH₄-responsive, contrary to the usual experience that they represent null mutations. This phenomenon may be caused by residual normal splicing with low efficiency or the generation of novel transcripts with in-frame insertions or deletions compatible with residual enzyme function [Jennings et al., 2000]. Nevertheless, it remains to be seen whether BH₄ sensitivity associated with splicing mutations can be confirmed in other patients and how this effect is mediated by BH₄.

PAH Database and BH₄-Responsiveness

Based on the information in the BIOPKUdb, mutations tabulated in the PAHdb were assigned as BH₄-responsive, nonresponsive, or unclear (see Materials and Methods for the definition) and predicted frequencies are calculated using the Hardy-Weinberg equation.

The 3,173 mutations in the PAHdb were classified into responsive alleles (32%) and nonresponsive alleles (68%, including unclear and mutations not found in the BIOPKUdb). For 633 patients, genotypes were defined and it was found that within this group of patients responsive alleles make up 36% of the mutations (Table 3). Study of the genotypic distribution revealed that out of 633 patients, 355 (56%) carried either one or two responsive alleles and thus are potential BH₄-responders. Based on the allele frequency (AF), we calculated the theoretical distribution of BH₄-responsive patients using the Hardy-Weinberg equation to be 58%, which corresponds well with the result of the genotype analysis (Table 4).

BH₄-Responsiveness in Europe, Northern China, and South Korea

The data from Germany and Northern Ireland contained genotype information, those from the remaining European countries only allele frequencies.

Based on the genotype information, 159 out of 275 (58%) potentially responsive patients were counted in Germany and 78 out of 119 (73%) in Northern Ireland. Using the Hardy-Weinberg formula a similar distribution was observed: 58% BH₄-responders in Germany and 76% in Northern Ireland (Table 4). The

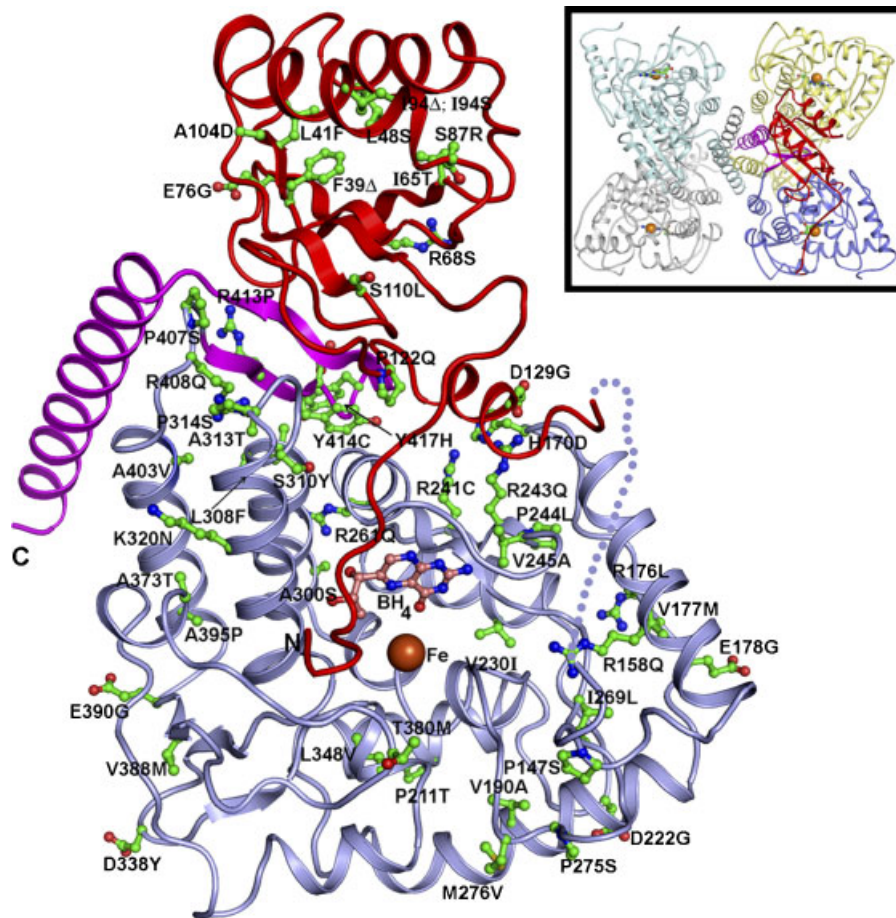


FIGURE 1. BH₄-responsive mutations found in PKU patients are mapped in the 3D crystal structure of the PAH monomer. In the active site, the iron atom and BH₄ cofactor are shown in red. The N-terminus starting over the active site as well as the rest of the regulatory domain are highlighted in red; catalytic domain in blue; and tetramer domain is in purple. In the top right corner is the native tetramer form of the enzyme.

calculation of the expected proportion of potentially responsive patients using the Hardy-Weinberg formula gave greatly different results ranging from 17% in the Baltic countries (Estonia, Latvia, and Lithuania) and Poland to a high 79% in Spain. Relative lower numbers of potentially responsive patients were found in Eastern Europe, mainly due to the high frequency of severe PKU mutations, in particular p.R408W, while higher numbers of responders are expected in the genetically heterogeneous Southern Europe populations. The average of potentially responsive patients in Europe was estimated to be 55% (Table 3). Data from Northern China comprised 155 fully defined genotypes with 310 alleles of which 106 were assigned as responsive according to our classification (Table 3). Approximately 54% of the genotypes are found to be potentially BH₄-responsive (57% using the Hardy-Weinberg formula) (Table 4). A total of 58 patients from South Korea with fully characterized mutations were used to calculate the expected frequency of BH₄-responsiveness. We found 38 out of 116 (33%) of responsive alleles (Table 3), which leads to 31 out of 58 (53%) potentially BH₄-responsive patients (55% according to the Hardy-Weinberg formula) (Table 4).

Genotype-Phenotype Correlation

Most patients tabulated in the BIOPKUdb belong to the group of MHP (42%) or mild PKU (37%). As classical PKU is frequently associated with homozygosity or compound heterozygosity for null mutations it is not surprising to find only 7% of moderate PKU and

13% of classic PKU in the BIOPKUdb. Of 173 patients with only one responsive allele 39% are MHP and 38% mild PKU, while of 134 patients with two responsive alleles (either homozygote or compound heterozygote) 47% are MHP and 35% mild PKU. Only 14% of the patients with one responsive allele and 10% of the patients with two responsive alleles belong to classic PKU.

We compared the BIOPKUdb information with two studies performed in PKU patients and faced a similar problem. Based on the loading test performed in 74 PKU patients, Fiori et al. [2005] detected 85% of patients to be BH₄ responders. Using the genotype analysis we found five more responders (p.R261Q/p.L48S, p.L48S/p.L48S, p.L48S/p.L48S, p.L48S/p.R408W, and p.R261Q/p.R53H), raising predicted BH₄-responsiveness to 92% (+7%). Perez-Duenas et al. [2004] loaded 42 PKU patients with BH₄ and found 44% of them to be responders. We confirmed BH₄-responsiveness in 18 out of 20 patients who were initially defined as responders; two were nonresponders according to the genotype (IVS2+5G>C/p.P362T and IVS4+5G>T/IVS4+5G>T). Out of the 22 initially assigned nonresponders, 11 were BH₄-responders according to the genotype information (p.R261Q/p.S349P, p.R243Q/p.I65T, p.L48S/p.S349P, IVS4+5G>T/p.R158Q, p.R261Q/p.R243X, p.V388M/p.S349P, IVS1+5G>T/p.R158Q, p.I65T/p.I65T, IVS4+5G>T/p.R243Q, p.I65T/IVS8+1G>A, and p.V388T/p.P279fsdelC). Thus, according to the BIOPKUdb classification by genotype, 69% (+25%) of patients described in this study are expected to be responders.

DISCUSSION

We tabulated biochemical and molecular data from 315 patients with BH₄-responsive PAH deficiency in the BIOPKUdb. Alleles and genotypes defined as BH₄-responsive were compared with data previously reported for PKU patients in some European countries, in Northern China, and in South Korea. Furthermore, the PAHdb knowledgebase compiling 3,171 mutant alleles from all

over the world was analyzed using the BIOPKUdb information. Comparison of different databases with the BIOPKUdb allowed us to estimate both allele and genotype frequencies of BH₄-responsiveness and to compare them with data obtained from BH₄ loading tests.

Kure et al. [1999] proposed that the mechanism of BH₄-responsiveness may be explained by distinct mutations in the PAH gene and that the composition of the PAH subunits may be critical for BH₄-responsiveness. Based on the BIOPKUdb information, we estimated that about 90% of BH₄-responsive PAH alleles are producing heterotetramers and depending on the type of mutation (truncated or not), the ratio between hetero- and homopolymers may be the predicting factor for the responsiveness.

Erlandsen and Stevens [2001] calculated the location and proximity of some of the mutations to the BH₄-binding site in the three-dimensional structure of PAH and predicted that some of them result in mutant enzymes that are K_m variants (with a lower binding affinity for BH₄) compared to the normal phenotype. The p.V388M mutation (BIOPKUdb AF 2.2%) was shown to result in a kinetic variant form of PAH by increasing K_m values for BH₄ from 22 μM to 82 μM [Leandro et al., 2000]. Further BIOPKUdb mutations reported with decreased binding affinity for BH₄ are p.F39L (AF 0.8%), p.I65T (AF 2.9%), p.R68S (AF 0.8%), p.I29G (AF 0.2%), p.P244L (AF 0.2%), p.L308F (AF 0.2%), and p.A309V (AF 0.5%) [Aguado et al., 2007; Perez et al., 2005].

The second and most probably major mechanism proposed for BH₄-responsiveness, a PAH-stabilizing effect of BH₄ (chaperon-like activity), has been confirmed by a number of investigations [Aguado et al., 2006; Erlandsen et al., 2004; Pey et al., 2004; Thöny et al., 2004]. BH₄ can prevent mutant PAH from both misfolding and inactivation. This has been shown for several common BH₄-responsive mutations: e.g., p.R261Q (AF 7.8%), p.Y414C (AF 7.0%), p.I65T (AF 2.9%), p.V388M (AF 2.2%), p.R68S (AF 0.8%), p.A309V (AF 0.5%), and p.P244L (AF 0.2%) [Pey et al., 2004]. In addition, BH₄ is able to enhance the wild-type enzyme activity without affecting PAH gene expression [Thöny et al., 2004; Scavelli et al., 2005]. A similar effect of BH₄ on the wild-type PAH activity was demonstrated by

TABLE 2. Distribution of BIOPKUdb Mutations in Different PAH Domains

Domain	Number of alleles	Percentage alleles	Number of different genotypes
All alleles			
Catalytic	401	63.7	66
Regulatory	90	14.3	26
Tetramerization	63	10.0	5
Intronic	75	11.9	17
Regulatory/ catalytic	1 ^a	0.2	1 ^a
Total	630	100	115
Responsive			
Catalytic	300	67.9	36
Regulatory	72	16.3	15
Tetramerization	62	14.0	4
Intronic	8	1.8	2
Regulatory/ catalytic	0	0.0	0
Total	442	100	57
Nonresponsive			
Catalytic	86	84.3	17
Regulatory	1	1.0	1
Tetramerization	1	1.0	1
Intronic	14	13.7	1
Regulatory/ catalytic	0	0.0	0
Total	102	100	20
Unclear			
Catalytic	15	17.4	13
Regulatory	17	19.8	10
Tetramerization	0	0.0	0
Intronic	53	61.6	13
Regulatory/ catalytic	1 ^a	1.2	1 ^a
Total	86	100	37

^aPatient with two mutations on the same allele.

TABLE 3. Allele Frequencies and BH₄-Responsiveness in Different Countries and Within PAHdb

Database ^a	All alleles	Responsive	Unclear	Non-responsive	Not in the BIOPKUdb	Potentially responsive patients (%) ^b
BIOPKUdb	630	473	55	102	—	315 (100)
PAHdb alleles	3,173	1,179	152	796	1,046	960 (61)
PAHdb genotypes	1,266	508	46	456	256	406 (64)
Northern Ireland (NI)	238	123	2	88	25	91 (77)
South Korea	116	38	17	26	35	32 (55)
Northern China	310	106	13	49	142	88 (57)
Europe	3,655	1,401	45	2,023	186	1,132 (62)
Germany	550	241	16	220	73	188 (68)
Spain	177	136	2	39	0	84 (95)
Sicily	108	81	1	26	0	51 (94)
France	186	106	2	78	0	76 (82)
Bulgaria	46	22	0	24	0	17 (73)
The Netherlands	39	17	0	22	0	13 (68)
UK (without NI)	351	144	5	181	21	114 (65)
Belgium	195	77	9	109	0	62 (63)
Republic of Ireland	464	178	3	258	25	144 (62)
Croatia	67	23	1	43	0	19 (57)
Norway	201	67	1	99	34	56 (56)
Denmark	281	85	2	191	3	72 (51)
Czech Republic	213	40	1	171	1	36 (34)
Poland	176	25	0	151	0	23 (26)
Iceland	16	2	0	10	4	2 (23)
Romania	28	3	0	25	0	3 (20)
Baltic States	319	31	0	288	0	29 (18)

^aSource data adapted from the PAHdb (www.pahdb.mcgill.ca), Zschocke and Hoffmann [1999], and Zschocke [2003].

^bCalculated using Hardy-Weinberg equation.

TABLE 4. Estimated and Predicted BH₄-Responsive Genotype Frequencies in Different Countries and Within PAHdb

Database ^a	Number of patients	Number of estimated responsive patients (%)	Number of predicted responsive patients (%) ^b
Germany	275	159 (58)	158 (58)
Northern Ireland	119	87 (73)	90 (76)
South Korea	58	31 (53)	32 (55)
Northern China	155	84 (54)	88 (57)
PAHdb	633	355 (56)	370 (58)

^aSource data adapted from the PAHdb (www.pahdb.mcgill.ca), Zschocke and Hoffmann [1999], and Zschocke [2003].

^bCalculated using Hardy-Weinberg equation.

Kure et al. [2004]. They proposed that the responsiveness to BH₄ in patients with PAH deficiency is probably due to suboptimal physiological concentrations of BH₄ in hepatocytes and that enhancement of the residual activity by BH₄ supplementation may be associated with a wide range of mutations.

Initial information about the frequency of BH₄-responsiveness within patients with PAH deficiency originates from a large retrospective study of over 1,900 loading tests [Bernegger and Blau, 2002]. Of the patients with baseline phenylalanine levels of 120–400, 400–800, 800–1,200, 1,200–1,600, 1,600–2,200, and > 2,200 μmol/L; 65%, 74%, 33%, 17%, 0%, and 10% of patients, respectively, were defined as responders. This study, however, used data obtained with both the old 33% less active formulation of BH₄ and the new fully-active 6R-BH₄ compound. In another study a total of 557 patients diagnosed with HPA were loaded with the active 6R-BH₄ (20 mg/kg) and blood phenylalanine was monitored over 8 to 24 hr [Fiege and Blau, 2007]. The overall prevalence of BH₄-responsiveness within patients with PKU for blood phenylalanine reduction of 20%, 30%, 40%, and 50% was 48%, 38%, 31%, and 24%, respectively, using the 8-hr modus, and 55%, 46%, 41%, and 33%, respectively, using the 24-hr modus. Using the standard 30% cutoff, BH₄-responsiveness was similar regardless of the modality in patients with mild hyperphenylalaninemia (79% to 83% responders), mild PKU (49% to 60% responders), and classical PKU (7% to 10% responders). About 46% of all HPA patients were responders 24 hr postloading [Fiege and Blau, 2007]. Extending the loading test to 48 hr [Fiege et al., 2005] or to one week [Shintaku et al., 2004] may detect additional patients who need longer time to show the decreased phenylalanine levels. Several studies observed a similar high percentage of BH₄-responsiveness: 62% by Leuzzi et al. [2006], 46% by Mitchell et al. [2005], 85% by Fiori et al. [2005], 38% by Bélanger-Quintana et al. [2005], 58% by Matalon et al. [2005], 44% by Perez-Duenas et al. [2004], and 66% by Muntau et al. [2002].

Our findings reveal several features common to different patient groups; both the frequency of potentially BH₄-responsive alleles and genotypes is higher than initially assumed from the loading test studies; the BH₄-responsiveness is characterized by a substantial residual PAH activity of at least one mutant allele; most of the BH₄-responsive mutant alleles are located in the catalytic domain of the PAH subunit; and using the Hardy-Weinberg formula, BH₄-responsiveness is higher than the effective distribution of potentially BH₄-responsive genotypes.

The PAH locus knowledgebase PAHdb is the largest database with 3,173 alleles reported in patients with various degrees of HPA (approximately 28% MHP, 36% mild PKU, and 56% classic PKU). Based on the alleles and genotype information, about 58% of patients are potentially BH₄-responders, an estimate higher

than the information observed from the BH₄ loading test (46% responders in a population with a similar proportion of various HPA patients) [Fiege and Blau, 2007]. By comparing the estimated frequency of BH₄-responsiveness in different European countries, we found that BH₄-responsiveness is much higher (>75%) in southern regions of Europe with a high frequency of BH₄-responsive alleles p.R261Q, p.V388M, p.I65T, p.R158Q, or p.L48S, than in central Europe (50–70%), or in some eastern European countries (<40%) with frequent severe alleles p.R408W, p.R252W, or IVS12+1G>A [Zschocke, 2003] (Table 3). Similar frequencies of BH₄-responsiveness were also estimated for the northern part of China and for South Korea (50–60% responders).

Several authors reported inconsistency of BH₄-responsiveness within the same genotypes and questioned the genotype–phenotype correlation. In addition, it has been reported that some patients who responded in the single loading test did less well on a continuous supplementation with BH₄ [Lambruschini et al., 2005]. Inconsistency of BH₄-responsiveness in patients with the same genotype is frequently reported, particularly in patients harboring p.L48S, p.I65T, p.R158Q, p.R261Q, and p.Y414C mutations [Desviat et al., 2004; Fiori et al., 2005; Leuzzi et al., 2006; Muntau et al., 2002; Nielsen et al., 2005; Perez-Duenas et al., 2004; Yildirim et al., 2007]. It is also interesting that some splicing mutations are reported as BH₄-responsive (e.g., IVS4–5C>G, AF 0.8% and IVS10–3C>T, AF 0.5%). Although, as already mentioned in some of them splicing may be affected only to a minor extent and/or resulting in a partially active expressed proteins, other factors like dietary fluctuation during the loading test and spontaneous reduction of phenylalanine concentrations may be responsible for apparent responsiveness.

In summary, from the evaluation of the data contained in the BIOPKUdb we would expect BH₄-responsiveness in about 50% of all patients with PAH deficiency. As discussed above, this number may vary from country to country and is higher than the BH₄ loading test data. It also needs to be shown to what extent genotype information can be used to predict *in vivo* BH₄-responsiveness. Different factors such as initial blood Phe levels, age of the patient, BH₄ pharmacokinetics, the length of time of the BH₄ test, and possibly BH₄ stabilizers like ascorbic acid or a second mutation on the same allele may influence the outcome of the test. Thus, although there is no absolute genotype–phenotype correlation, mutation analysis provides useful information on potential nonresponders in patients harboring two null alleles and may, to some extent, predict possible BH₄-responders. Further investigations are necessary in order to estimate the optimal BH₄ loading test conditions (amount of BH₄ and time of blood Phe monitoring), and genotype analysis may help to select target populations.

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